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(71) Applicant *(for all designated States except US)*: ISIS INNOVATION LIMITED [GB/GB]; 2 South Parks Road, Oxford 0X1 3UB (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)* : WAKEFIELD, Ann, Elizabeth [GB/GB]; Park View, Woodstock Road, Charlbury, Oxford 0X7 3ET (GB). HOPKIN, Julian, Meurglyn [GB/GB]; 20B Plantation Road, Oxford 0X2 6JD (GB). MOXON, Edward, Richard [GB/GB]; 17 Moreton Road, Oxford 0X2 7AX (GB).

(74) Agent: PENNANT, Pyers; Stevens, Hewlett & Perkins, Serjeants' Inn, Fleet Street, London EC4Y 1LL (GB).

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5' - G A T G G C T G T T T C C A A G C C C A - 3'
5' - G T G T A C G T T G C A A A G T A C T C - 3'
(I)

(57) Abstract

A method of assaying a sample of DNA from respiratory secretion of a patient for *Pneumocystis carinii*, comprises ampli fying a polynucleotide sequence derived from *P. carinii* by a polymerase chain reaction, and detecting the amplified sequence i present. Two DNA sequences are given, together with a number of pairs of oligonucleotide primers, including particularly th pair (I).

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DNA FOR DIAGNOSING PNEUMOCYSTIS CARINII

Introduction

Pneumocystis carinii is established as the prime cause of opportunistic pneumonia in patients with AIDS and those immunosuppressed on oncology and transplant units. Debate over the taxonomy of P.carinii continues and the fastidious nature of the parasite still demands the use of microscopy after histochemical staining or immunofluorescence for the detection of the parasite in diverse forms of lung samplings [1,2]. The requirement for observer expertise is significant and limits the diagnostic power of these techniques. There are sound theoretical grounds for believing that DNA amplification using the polymerase chain reaction (PCR) [3] might provide both a specific and sensitive means of identifying the parasite in clinical samplings and one that finally might be amenable to automation. Other applications of DNA amplification to the study of the epidemiology of P.carinii infection are also evident.

European Patent Specification 327390 describes DNA sequences produced by recombinant DNA technology from an experimental rat model which hybridise to DNA of P.carinii but not to mammalian DNA. These DNA sequences were present as inserts in plasmids of which two were designated pAZ 102 and pAZ 112. This invention results from further work described herein:-

A. We have sequenced the inserts of pAZ 102 and pAZ 112. The sequences are set out in Figures 1 and 3.

B. Using suitable oligonucleotide primers we have amplified, by the polymerase chain reaction (PCR) technique, P.carinii DNA from infected rat lung samplings, to an extent that the amplified DNA was

easily detectable by staining an electrophoresis gel. In the same way, we have amplified P.carinii DNA from infected human lung samplings.

C. Analysis of the amplified DNA from human samples has shown significant sequence differences from the infected rat material. The human sequence is set out in Figure 1, and the similarities and differences between human and rat sequences are highlighted.

D. The human sequence, leads to improved oligonucleotide primers which more efficiently amplify P.carinii DNA of human origin.

E. We have developed a more sensitive detection system, involving hybridising the amplified DNA to a labelled probe which probe is part of the sequence determined in C. intermediate the two primers. By these means we are able to detect P.carinii DNA, and thus to diagnose infection by P.carinii, in patients who do not (yet) show clinical symptoms.

In one aspect, this invention provides, as new chemical compounds, the nucleic acid sequences shown in Figures 1 and 3, single and double chain fragments thereof at least 15 nucleotides in length, and nucleic acid sequences and fragments having at least 90% homology thereto. These result from steps A. and C. above.

In another aspect, the invention provides a method of assaying a sample of DNA from respiratory secretion of a patient possibly infected with Pneumocystis carinii, which method comprises using a pair of oligonucleotide primers based on the sequences shown in Figure 1 or Figure 3 to amplify by a polymerase chain reaction a polynucleotide sequence derived from P.carinii if such sequence is present in the sample, and detecting the amplified sequence if present. This is step B. above, and steps D. and E. constitute preferred features of the method.

For maximum efficiency and specificity of the PCR reaction, the choice of oligonucleotide primers is critical. The primers must be based on the sequence to be amplified and may be identical to the two ends. However, identity is by no means essential (R. Sommer and D. Tautz, Nucleic Acids Research, Vol. 17, No. 16, 1989, p. 6749). Generally the two or three nucleotides at the 3'-end of the primer, and at least 50% (preferably at least 90%) of all the nucleotides of the primer, are homologous to the sequence to be amplified.

The primers are partly or completely homologous to particular sites of the sequence to be amplified. For maximum efficiency of the PCR reaction, the location of those sites is also important. Although most primers will work with varying degrees of success, general guidelines for obtaining useful primers are found in the literature (see Saiki R. K. et al., The Polymerase Chain reaction in Genome Analysis (Ed K. E. Davies) IRL, Oxford, 1988). However, the design of effective primers tends to be empirical. Described below are one pair of primers derived from pAZ 102 that have proved outstanding; and several pairs of primers derived from pAZ 112 that have proved effective.

In other respects, the PCR conditions may be conventional. The primers may be at least 8, conveniently about 20, nucleotides in length. The number of cycles required to achieve sufficient amplification may be from 15 to 50. If required to improve specificity, two different pairs of primers may be used. The resulting amplified sequence has a predetermined length, and moves a predetermined distance on an electrophoresis gel. The resulting band can be visualised, either by conventional staining techniques, or by hybridisation to a labelled probe which probe is homologous to part or all of the known

sequence being amplified.

Reference is directed to the accompanying Figures, in which:-

Figure 1 comprises sequence data on different DNA samples. Row 1 entitled "Rat" is from lung samplings from a rat infected with P.carinii. Row 2 entitled "Human" is from lung samplings of infected humans. Secondary structure has been taken into consideration and gaps (-) introduced to obtain maximum alignment. Numerous differences between human and rat sequences are shown boxed.

Figure 2 is a diagram of the circular plasmid pAZ 112 showing certain features including the positions of polynucleotide primers used in PCR.

Figure 3 comprises the complete sequence of the insert of pAZ 112, with the oligonucleotide primers marked. R/C means reversed and complemented, i.e. the actual sequence of the primer is the reverse and complementary to that marked.

Table 2 lists the oligonucleotide primers referred to in Figures 2 and 3.

Table 3 lists the primer combinations successfully used by us and the approximate size of the resulting amplification product.

The following Examples illustrate the invention. Example 1 relates to DNA from the plasmid pAZ 102 whose sequence is shown in Figure 1. Example 2 relates to DNA from the plasmid pAZ 112 whose sequence is shown in Figure 3. Example 3 reports a clinical trial following the method of Example 1.

Example 1

Methods

Cloning and sequencing of part of the gene coding for the large sub-unit of the mitochondrial ribosomal RNA from P. carinii

P. carinii pneumonia was induced in the rat model and DNA extracted and cloned from a parasite enriched fraction as previously described [4]. P.carinii specific sequences were confirmed by characteristic in situ hybridisation patterns and recombinant plasmid pAZ102 was selected as a candidate mitochondrial sequence because of strong signals derived in dot blot hybridisation studies on infected samples. The recombinant plasmid pAZ102 (insert 570 bp) was sequenced using Sanger's chain termination method and the Sequenase kit (United States Biochemical Corporation, Cleveland, USA), ^{35}S(Amersham, UK), Sequagel (National Diagnostics, Manville, USA). The DNA sequence was compared with those available in several databases including EMBL and Genbank. From the sequence data on pAZ102 and comparative analysis of the databases, the fragment was identified as a portion of the gene coding for the large sub-unit of the mitochondrial, ribosomal RNA of P.carinii and this showed significant homology with fungal sequences (manuscript in preparation).

Oligonucleotide primers

Two sequences of moderate conservation that were specific to P.carinii were selected for construction of oligonucleotide primers for the polymerase chain reaction:- pAZ102 - E:-5'-GATGGCTGTTTCCAAGCCCA-3'; pAZ102-H:-5'-GTGTACGTTGCAAAGTACTC-3'. An oligonucleotide for confirmatory Southern hybridisation on amplification products was chosen, pAZ102-L1. Subsequently a new internal oligonucleotide specific to human P.carinii sequences was constructed, pAZ102-L2 (Table 1).

Template DNA

i) Samplings for DNA amplification using our oligonucleotide primers comprised a) pulmonary lavage samplings from 3 humans and 3 rats with P.carinii pneumonia documented by methenamine silver staining and microscopy, and b) isolates from a series of organisms including some

potential pulmonary pathogens: Candida (an albicans and a non-albicans strain), Cryptococcus neoformans, Mycobacterium tuberculosis, Saccharomyces cerevisiae and Aspergillus nidulans.

ii) Template DNA was prepared from each sample by proteinase K digestion in the presence of SDS and EDTA followed by phenol/chloroform/ether extraction, and ethanol precipitation.

DNA amplification

Using primers pAZ102-E and pAZ102-H the template samples, together with control samples without template underwent 40 cycles of amplification performed with denaturation at 94°C for 90 seconds, annealing at 50°C for 90 seconds and extension at 72°C for two minutes (Techne, UK). The DNA amplification reaction mixture (50 µl) contained 50mM KCl, 10 mM Tris, pH 8, 0.01% (w/v) gelatin, 3mM $MgCl_2$, 400 µM dNTPs (Boehringer Mannheim, UK), 0.4-1.0 µM oligonucleotide primer and 3 units of Amplitaq (Perkin Elmer Cetus, UK).

To avoid the possibility of false negative results in the human clinical samples, i.e. failure to detect the specific amplification product for technical reasons, we carried out a parallel polymerase chain reaction on each sample using primers derived from the human anti-thrombin gene, exon 2 These primers, (AT1: -5'-GTTGCAGCCTAGCTTAACTTGGCA-3'; AT4: -5'-GGTTGAGGAATCATTGGACTTG-3') allowed amplification to take place using human genomic DNA as template. In each of the clinical samples the 500 bp specific product was detected, demonstrating efficient amplification.

The potential problem of contamination in PCR was monitored by systematic use of the following techniques: i) including several negative control samples with no added template DNA; ii) by the use of UV-irradiation of the PCR reaction mixes prior to the addition of the template DNA [5]. iii) the use of separate disposable microcapillaries for the addition of each template (Laser Laboratory Systems Ltd, UK). The control

samples remained negative in all experiments.

Amplified products (10 µl) were electrophoresed in 1.5% agarose gels and visualised after ethidium bromide staining by ultraviolet light. The gel was Southern blotted on to Hybond N (Amersham, UK) and hybridised with ^{32}P end-labelled internal primer at 46°C (pAZ102-L1) or 40°C (pAZ102-L2) for 3 hours[6]. Filters were subsequently washed at high stringency at 54°C (pAZ102-L1) or 48°C (pAZ102-L2) and filters exposed to radiographic film at -80°C with intensifying screens. The expected amplification product was 355bp long in the rat derived parasite, that from the human derived parasite being 9 bp shorter.

Sequencing of products of DNA amplification

The PCR product was gel purified and recovered from the agarose gel using Geneclean (Bio 101, Inc). The purified DNA was heat denatured and sequenced as described above using primers pAZ102-H or pAZ102-E at 20pmole/µl.

Results

The oligonucleotide primers derived from rat P.carinii produced efficient amplification of specific sequence from both rat and human hosts, shown by ethidium bromide staining but none from the range of other organisms including some potential pulmonary pathogens.

The internal oligonucleotide, pAZ102-L1, derived from the rat P.carinii, produced strong hybridisation signals on Southern hybridisation with amplified products from the infected rat lungs, but weak signal, at high stringency, with the amplified product derived from human samples although these were visible on ethidium bromide staining. Direct sequencing of the amplified products from each of the rat and human samples allowed comparison of their sequence and demonstrated limited but consistent differences between the P.carinii DNA from these two hosts which included 5 base changes in the sequence of the internal oligonucleotide pAZ102-L1 (Table 1).

An oligonucleotide specific to the human derived organisms was constructed, pAZ102-L2, which showed strong hybridisation with the amplified product from human P.carinii and conversely showed weak hybridisation with the rat P.carinii amplified product. It produced no hybridisation with the PCR products of the range of other organisms tested.

Studies using serial dilutions of human derived P.carinii template DNA indicated that the application of oligoblotting with pAZ102-L2 to amplified DNA products increased the sensitivity of detection by at least 100 fold over visualisation by ethidium bromide staining.

The P.carinii oligonucleotide primers successfully amplified specific PCR product from bronchoscopic alveolar lavage samplings from 10 HIV-positive individuals with pneumocystis pneumonia as documented by positive methenamine silver staining on the lavage samples. Lavage samples from 5 immunocompetent subjects were studied as controls. These failed to show specific PCR product by ethidium bromide staining or oligoblotting.

Discussion

We have characterised a portion of the gene coding for the large sub-unit of mitochondrial, ribosomal RNA from P.carinii and comparative analysis of this indicates significant homology with fungal sequences. This result accords with data we have on other mitochondrial genes and the observations of other groups on ribosomal RNA[7].

We have identified P.carinii specific sequences from which we have constructed oligonucleotide primers which allow efficient amplification of part of this ribosomal RNA gene from P.carinii infecting both rat and human hosts but not from a range of other organisms including some potential pulmonary pathogens. These results indicate the specificity of the amplified products to P.carinii, confirmed on Southern hybridisation with internal oligonucleotide pAZ102-L1 derived from the rat sequence and applied to the rat pulmonary samplings. Results from the human samplings suggested the likelihood of differences in sequence between the amplified products from the rat and human. This was confirmed by comparison of sequences which indicated limited, but consistent differences.

This finding is highly relevant to the hopes for developing DNA amplification as a diagnostic tool in clinical medicine. We have shown that our oligonucleotide primers can be used to identify the presence of P.carinii in a number of bronchoalveolar lavage samples. By using our second internal oligonucleotide, pAZ102-L2, which is specific to human P.carinii, the sensitivity of detection of amplified product is considerably increased. This method shows great potential for use on non-invasive samples such as induced sputum where parasite numbers are lower. It will not only be valuable in diagnosis but also in addressing questions relevant to the epidemiology of P.carinii.

The application of DNA amplification to diagnosis will require careful calibration to ensure that levels of P.carinii in keeping with clinical pneumonia can be distinguished from lesser degrees of colonisation that are likely to occur in the immunodeficient before clinical disease is manifest. Such methods of calibration of DNA amplification are becoming available [8,9] and their application to diagnostic studies on P.carinii in diverse clinical samplings, including lavage and induced sputum, are now required.

Example 2

For pAZ 112 we used the techniques described in Example 1, and the oligonucleotide primers given in Table 2 in the combination set out in Table 3. We achieved amplification by PCR of the sequences of pAZ 112 shown in Figure 3.

Example 3

Clinical Specimens

Alveolar lavage samples were obtained from 47 patients investigated by bronchoscopy at the Churchill Hospital, Oxford, and at the Middlesex Hospital, London.

Thirty seven patients were immunosuppressed, either by HIV infection (33) or by treatment for lymphoma (2), vasculitis (1) or leukaemia (1). All patients had symptoms of acute respiratory illness with one or more of the following features: abnormal chest signs, arterial hypoxaemia, or abnormal chest radiograph. The 10 remaining patients were immunocompetent, undergoing bronchoscopy to investigate various respiratory disorders. Routine microbiological and cytological analysis including methenamine silver staining was performed on each lavage and an aliquot reserved for the DNA amplification study, performed as described in Example 1.

Results

On the basis of clinical progress, response to treatment with nebulised pentamidine (20 cases) or cotrimoxazole (7 cases) and results of standard investigation including methenamine silver staining,

the 47 patients were eventually categorised into four groups (Table 4); 16 immunosuppressed patients with a positive diagnosis of pneumocystis pneumonia by silver staining on lavage and response to treatment, 6 immunosuppressed patients with clinical response to treatment, but negative silver stains on lavage, 15 immunosuppressed patients with neither response to treatment nor positive silver staining on lavage and in whom an alternative diagnosis to account for the respiratory disease was available in 12, and the 10 immunocompetent patients from the routine bronchoscopy list. The results of DNA amplification assayed by the visualisation of a 346 base pair DNA band after a) ethidium bromide staining and b) autoradiography after oligoblotting were compared with these clinical data categorisations (Table 4).

No P.carinii DNA was detectable in the samples from the immunocompetent group. All of the 16 immunosuppressed individuals with P.carinii identified by methenamine silver staining on alveolar lavage had amplified P.carinii DNA visible by both ethidium bromide staining and oligoblotting. Of the 6 individuals judged to have had pneumocystis pneumonia by clinical symptoms and response to treatment but not confirmed by identification of parasites in methenamine silver stained lavage samples, 4 were positive using DNA amplification - both by ethidium bromide staining and oligoblotting - and 2 negative by both methods.

Lesser degree of P.carinii infection were detected in the oligoblots - but not by ethidium bromide staining - of the DNA amplification product in lavage samples from 3/15 of the immunosuppressed subjects without pneumocystis pneumonia. The intensity of the signal was less than that obtained from patients with acute P.carinii infection but significantly greater than a barely visible signal obtained in 4

other samples from this patient group, and from 3 of 12 preliminary washings of the bronchoscope after routine cleaning and sterilisation.

Example 4

The method of Example 1 was extended to test induced sputum samples. Fifty one episodes of acute respiratory illness in immunosuppressed HIV-infected individuals were studied. Bronchoscopic alveolar lavage was obtained from each patient and in thirty seven instances induced sputum was also obtained. Samples were examined by routine microbiological and cytological methods, including methenamine silver staining for P.carinii; a part of each sample was reserved for DNA amplification. DNA was extracted from 1 ml lavage or sputum by proteinase K digestion (1 mg/ml final concentration of proteinase K, in the presence of 10 mmol EDTA, pH 8.0 and 1% weight/volume sodiumdodecylsulphate, at 50°C for 16 hours and phenol/chloroform extraction. DNA amplification was done with the oligonucleotide primers pAZ102-E and pAZ102-H, with denaturation at 94°C for 90 s, annealing at 55°C for 90 s, and extension at 72°C for 2 min (40 cycles). The amplification products were subjected to electrophoresis in 1.5% agarose gel and the specific P.carinii sequence (346 base pairs) was identified by visualisation with ultraviolet light after ethidium bromide staining or by oligohybridisation, after Southern transfer and autoradiography with the internal primer pAZ102-L2. Scoring of the DNA bands was done without knowledge of the results of silver staining or of final clinical diagnosis, which was assessed by clinical features and response to treatment with co-trimoxazole or pentamidine.

The results from the paired lavage and sputum

samples are summarised in the table 5 below. All 14 patients who had a final clinical diagnosis of pneumocystis pneumonia and who also had a positive silver stain on lavage, had a strong signal of amplified pneumocystis DNA from both the lavage sample and sputum. In 5 other patients with a final clinical diagnosis of pneumocystis pneumonia but negative silver stains, 4 were strongly positive by DNA amplification in alveolar lavage; 3 of these 4 were also positive in induced sputum. Silver stain was positive in only one third of sputum samples from cases of pneumocystis pneumonia.

Table 5

Final clinical diagnosis (numbers)	DNA amplification positive		silver stain positive	
	sputum	lavage	sputum	lavage
Pneumocystis pneumonia (20)	18	19	7	14
Other diagnoses (17)	1	1	0	0

Positive signals of amplified DNA can be categorised as strong (visible after ethidium bromide staining of the agarose gel) or weak (visible only on autoradiography after oligoblotting). Independent calibration experiments have shown that a strong signal points to 100 organisms or more in a sample, whereas a weak signal indicates from 1-2 organisms up to 100 organisms per sample. In broad terms, it may be said that patients providing samples with strong signals show clinical symptoms of pneumocystis pneumonia,

whereas patients providing samples with weak signals are in the pre-clinical stage. Thus, in 20 cases judged to have clinical pneumocystis pneumonia, a strong DNA amplification signal was obtained in 19 (95%) of the lavage samples and in 18 (90%) of the paired sputum samples. By contrast, microscopy after silver staining could only diagnose 35% of these cases on induced sputum. The sensitivity of the DNA method is therefore excellent; it is unlikely that the single case, negative by both DNA amplification and silver staining on lavage, had pneumocystis pneumonia.

The specificity of DNA amplification may be judged from the results of another study involving 44 patients, in whom the final clinical diagnosis was of another respiratory illness (i.e. not pneumocystis pneumonia). A strong amplification signal was obtained both in the lavage and sputum samples in only one of these 44 patients; this patient had had a previous episode of pneumocystis pneumonia and returned with a further documented episode within ten weeks of the current study.

Table 1

Comparative sequence of oligonucleotides pAZ102-L1 (rat P. carinii) and pAZ102-L2 (human P. carinii).

pAZ102-L1	5' -	A	T	A	A	G	G	T	G	A	G	G	A	G	T	C	G	A	G	A	G	-	3'
pAZ102-L2	5' -	A	T	A	A	G	G	T	A	G	A	T	A	G	T	C	G	A	A	A	G	-	3'

Table 2

pAZ 112 - Oligos used in PCR

Name	Sequence	Length mer
1F	A G A A C T G G A T T C T T A G A	17
1R	A G A A G T A T C A A G T T G A T	17
9F	C T C A T G C T T C A A G T G A C	17
3F	C A C T T T T A C T A A T A G C C	17
3R	T T G A T T A T C C A A C C A A G A	18
4F	T A A A T C C A C A T T C A A A G	17
4R	T G T T T T T A G T T A A C C C T	17
5F	T A C G G G A T T G A G A T A A T	17
5R	T T T A T G A T G G A G T A C C A	17
6F	T A T T T G G A A T T G G A T G A	17
6R	T C T T T G C C T T G T T A G G A	17
10F	T A G A C G G T C A C A G A G A T C A G	20
10R	G A A C G A T T A C T A G C A A T T C C	20

Table 3

PCR Results for pAZ 112

Oligos	Approx. Size of Amplification Product, bp
1F + 9F	285
1F + 1R	610
3F + 3R	516
4F + 4R	198
5F + 5R	183
6F + 6R	148
10F + 10R	700

Table 4

Results of DNA amplification of P. carinii DNA in bronchoalveolar lavage samples; comparison with methenamine silver staining and clinical response to treatment

Patient group (Number of Patients)	Silver Stain	Response to treatment	Positive DNA amplification for P. carinii		Negative DNA amplification for P. carinii
			ethidium bromide stain + Oligoblot	Oligoblot alone	
Immunosuppressed (16)	+	+	16	0	0
(6)	-	+	4	0	2
(15)	-	-	0	3 †	12 ‡
Immunocompetent (10)	-	-	0	0	10

† Alternative diagnosis: Toxoplasma gondii; Mycobacterium avium intracellulare; pulmonary lymphoma

‡ Alternative diagnosis: Endobronchial Kaposi's sarcoma (2), Mycobacterium avium intracellulare (2), Mycobacterium avium intracellulare and cytomegalovirus (1), Salmonella typhimurium (1), Pseudomonas putida (1), Streptococcus pneumonia and Haemophilus influenzae(1), bronchiectasis(1), no diagnosis (3)

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CLAIMS

1. The nucleic acid sequences shown in Figure 1, single and double chain fragments thereof at least 15 nucleotides in length, and nucleic acid sequences and fragments having at least 90% homology thereto.

2. The nucleic acid sequence shown in Figure 3, single and double chain fragments thereof at least 15 nucleotides in length, and nucleic acid sequences and fragments having at least 90% homology thereto.

3. Peptide sequences transcribed from the nucleic acid sequences claimed in Claim 1 or Claim 2.

4. A method of assaying a sample of DNA from respiratory secretion of a patient possibly infected with Pneumocystis carinii, which method comprises using a pair of oligonucleotide primers based on the sequences shown in Figure 1 or Figure 3 to amplify by a polymerase chain reaction a polynucleotide sequence derived from P.carinii if such sequence is present in the sample, and detecting the amplified sequence if present.

5. A method as claimed in Claim 4, wherein the amplified sequence is detected by electrophoresis and staining.

6. A method as claimed in Claim 4, wherein the amplified sequence is detected by hybridisation to a labelled probe which probe is a nucleic acid sequence according to Claim 1 or Claim 2.

7. A method as claimed in Claim 7, wherein the probe is the 20-mer.

5'- A T A A G G T A G A T A G T C G A A A G - 3'

8. A method as claimed in any one of Claims 4 to 7, wherein the oligonucleotide primers are:-

5' - G A T G G C T G T T T C C A A G C C C A - 3'

5' - G T G T A C G T T G C A A A G T A C T C - 3'.

9. A method as claimed in any one of claims 4 to 8, wherein the respiratory secretion assayed is induced sputum.

FIG.1

Rat TTGTGGTAAGGAGTGAAATACAAATCGGACTAGGATATAGCTGGTTTTCTGCGAAATG
Human TTGTGGTAAGGAGTGAAATACAAATCGGACTAGGATATAGCTGGTTTTCTGCGAAAAT
AGAAG
AGAAG

FIG.2

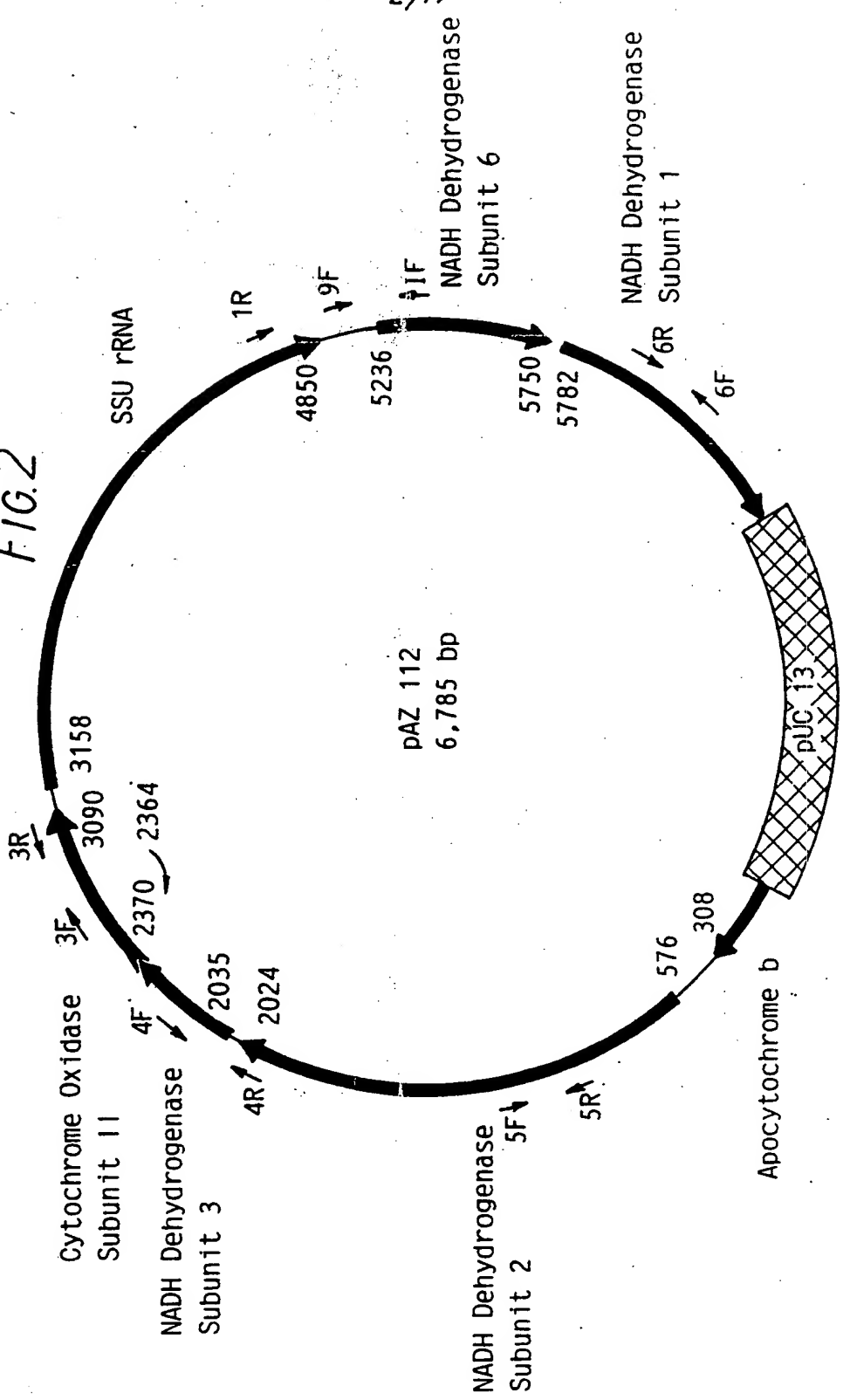
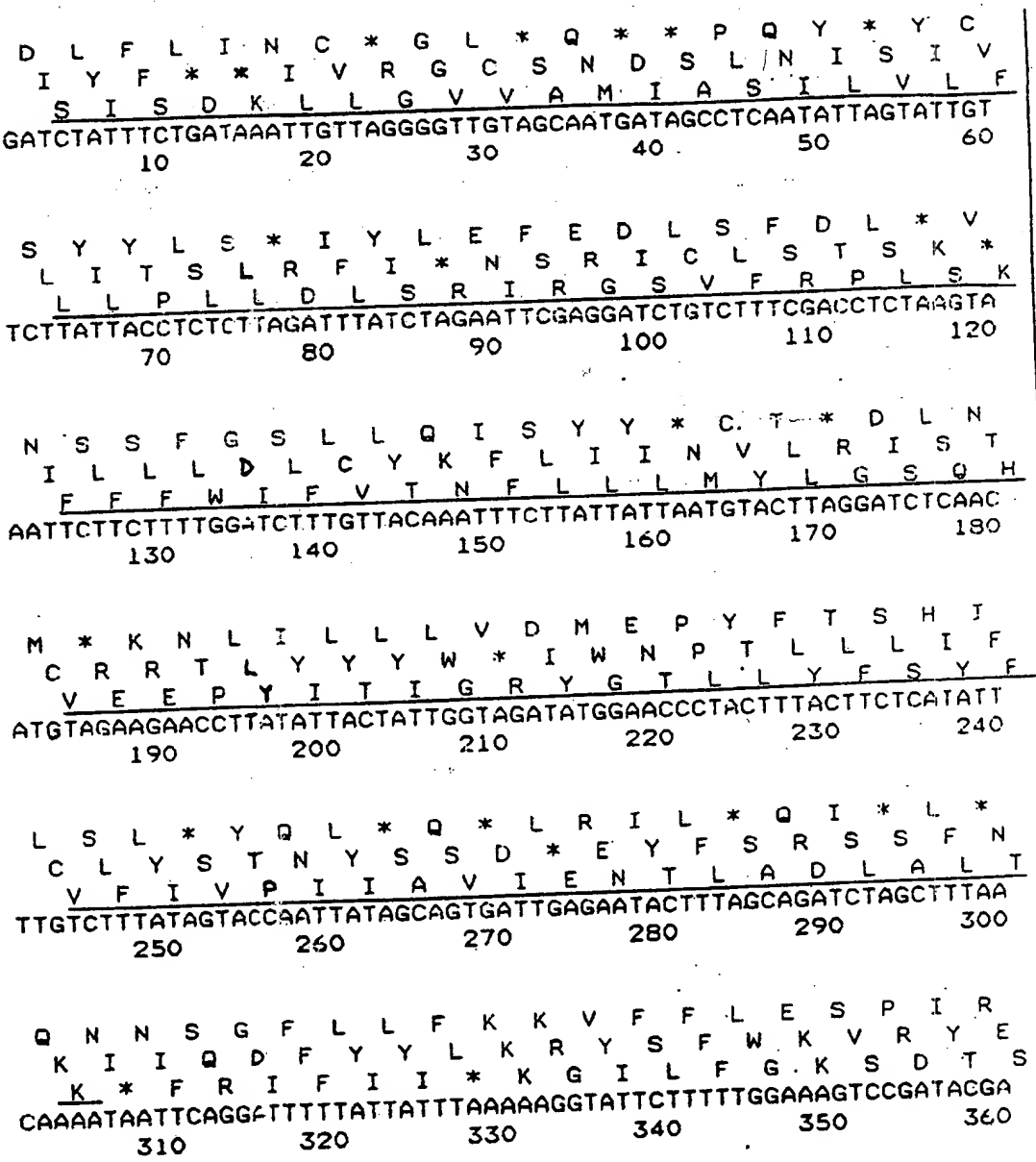

pAZ 112
6,785 bp
SSU rRNA
1R
9F
1F
NADH Dehydrogenase Subunit 6
NADH Dehydrogenase Subunit 1
6R
6F
pUC 13
Apocytochrome b
5R
5F
NADH Dehydrogenase Subunit 2
4R
4F
NADH Dehydrogenase Subunit 3
Cytochrome Oxidase Subunit II
3F
3R
4850
5236
5750
5782
308
576
2024
2035
2370
2364
3090
3158

FIG.3



Apocytochrome b

SUBSTITUTE SHEET

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 L K I I E * N L F C K N R R H L E E T K
  * R * * N K I Y S V K I E D I * K K L K
GTTAAAGATAATAGAATAAAATCTATTCTGTAAAAATAGAAGACATTTAGAAGAAACTAA
      370       380       390       400       410       420

N L Y L * M E L S E P I N L I F V I Y *
 I S I F R W S * A S L L I L S S L F T K
  S L S L D G V K R A Y * S Y L R Y L L N
AATCTCTATCTTTAGATGGAGTTAAGCGAGCCTATTAATCTTATCTTCGTTATTTACTAA
      430       440       450       460       470       480

T K * Y L N C L V F K L I S I D N * I I
 L S N I * I A W Y S S * F Q * I I K * L
  * V I F K L L G I Q V N F N R * L N N *
ACTAAGTAATATTTAAATTGCTTGGTATTCAAGTTAATTTCAATAGATAATTAAATAATT
      490       500       510       520       530       540

N K Q N S A Y * N L K K C Y Y Q V * L V
 I N R I R L I K I * K N V I I K Y N * S
  * T E F G L L K S E K M L L S S I I S C
AATAAACAGAATTCGGCTTATTAAAATCTGAAAAAATGTTATTATCAAGTATAATTAGTC
      550       560       570       580       590       600

S * L L * L F L L L T G I * F C * V E *
 A N C Y S S F F F S L E F S F V E * N K
  L I A I A L S S S H W N L V L L S R I S
AGCTAATTGCTATAGCTCTTTCTTCTTCTCACTGGAATTTAGTTTTGTTGAGTAGAATAA
      610       620       630       640       650       660

V L F P * S I L * F * H I M F I M * R L
 Y Y F L N L F Y N F D I * C L L C R D Y
  I I S L I Y S I I L T Y N V Y Y V E I I
GTATTATTTCCTTAATCTATTCTATAATTTTGACATATAATGTTTATTATGTAGAGATTA
      670       680       690       700       710       720

* G * V * E S I M D F Y K * L V * H N L
 R V R F R N L * W I F T S N * F N T I C
  G L G L G I Y N G F L Q V T S L T Q F V
TAGGGTTAGGTTTAGGAATCTATAATGGATTTTTACAAGTAACTAGTTTAACACAATTTG
      730       740       750       760       770       780

L I S L F F S * E F * Y W V L Q G S I M
 * Y L Y F S L R N F N I G Y Y R V L S C
  D I F I F L L G I L I L G I T G F Y H V
TTGATATCTTTATTTTTCTCTTAGGAATTTTAATATTGGGTATTACAGGGTTCTATCATG
      790       800       810       820       830       840
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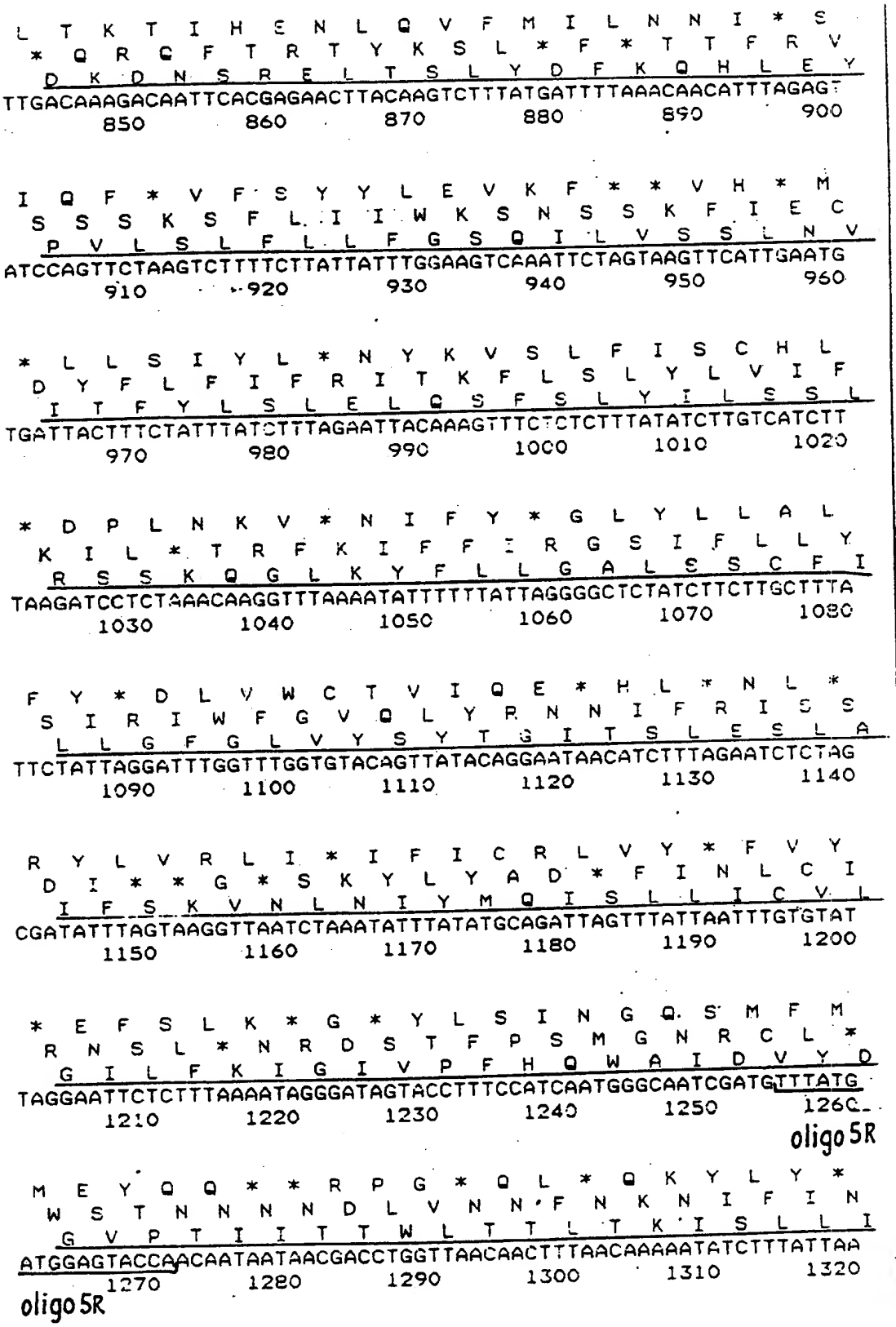
oligo 8F

NADH dehydrogenase subunit 2



NADH dehydrogenase subunit 2

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      Y  S  *  W  S  L  F  I  I  I  H  Q  K  I  G  Q  Q  Y  *  C
      I  L  N  G  V  Y  L  S  S  F  I  R  K  L  D  N  N  I  N  V
      F  L  M  E  F  I  Y  H  H  S  S  E  N  W  T  T  I  L  M  L
TATTCTTAATGGAGTTTATTTATCATCATTCATCAGAAAATTGGACAACAATATTAATGT
   1330       1340       1350       1360       1370       1380

      Y  Y  Q  C  Y  L  *  *  W  G  L  S  W  D  Y  L  N  P  V  L
      I  I  S  V  I  C  D  S  G  V  S  P  G  I  I  S  I  P  Y  *
      L  S  V  L  S  V  I  V  G  S  L  L  G  L  S  Q  S  R  I  K
TATTATCAGTGTTATCTGTGATAGTGGGGTCTCTCCTGGGATTATCTCAATCCCGTATTA
   1390       1400       1410       1420       1430       1440
                                                 oligo 5F(R/C)

      N  D  Y  *  S  I  A  W  *  V  M  *  D  F  *  C  Y  P  Y  Q
      T  I  I  N  L  *  H  G  K  S  C  R  I  F  N  A  I  L  I  N
      R  L  L  I  Y  S  M  V  S  H  V  G  F  L  M  L  S  L  S  I
AACGATTATTAATCTATAGCATGGTAAGTCATGTAGGATTTTTAATGCTATCCTTATCAA
   1450       1460       1470       1480       1490       1500

      *  *  Q  R  N  L  *  K  H  S  Y  F  I  *  Y  N  I  V  *  Q
      N  D  R  E  I  F  R  S  I  L  I  L  F  S  T  I  *  Y  N  K
      M  T  E  K  S  L  E  A  F  L  F  Y  L  V  Q  Y  S  I  T  N
TAATGACAGAGAAATCTTTAGAAGCATTCTTATTTTATTTAGTACAATATAGTATAACAA
   1510       1520       1530       1540       1550       1560

      I  *  M  S  F  *  F  *  L  L  W  D  I  S  T  K  I  Q  I  V
      F  K  C  L  F  N  S  D  C  Y  G  I  F  L  Q  K  S  R  *  *
      L  N  V  F  L  I  L  I  A  M  G  Y  F  Y  K  N  P  D  S  E
ATTTAAATGTCTTTTTAATTCTGATTGCTATGGGATATTTCTACAAAAATCCAGATAGTG
   1570       1580       1590       1600       1610       1620

      K  I  L  Q  *  F  I  S  I  V  *  E  V  W  *  E  S  S  P  Y
      R  F  S  N  N  L  Y  Q  *  F  K  R  F  G  E  S  P  A  L  I
      D  S  P  I  I  Y  I  N  S  L  R  G  L  V  P  V  Q  P  L  L
AAGATTCTCCAATAATTTATATCAATAGTTTAAGAGGTTTGGTGAGAGTCCAGCCCTTAT
   1630       1640       1650       1660       1670       1680

      Y  L  F  V  *  P  S  L  Y  Y  L  W  G  E  Y  R  L  L  *  D
      I  Y  L  F  S  H  L  F  T  I  S  G  G  N  T  A  F  Y  R  I
      S  I  C  L  A  I  S  L  L  S  L  G  G  I  P  P  F  I  G  F
TATCTATTTGTTTAGCCATCTCTTTACTATCTCTGGGGGGAATACCGCCTTTTATAGGAT
   1690       1700       1710       1720       1730       1740

      F  L  V  S  *  I  F  Y  I  V  R  *  L  K  D  I  Y  L  Y  L
      F  W  *  V  K  Y  S  I  *  Y  D  N  S  R  I  F  I  Y  I  Y
      F  G  K  L  N  I  L  Y  S  T  I  T  Q  G  Y  L  F  I  S  I
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   1750       1760       1770       1780       1790       1800
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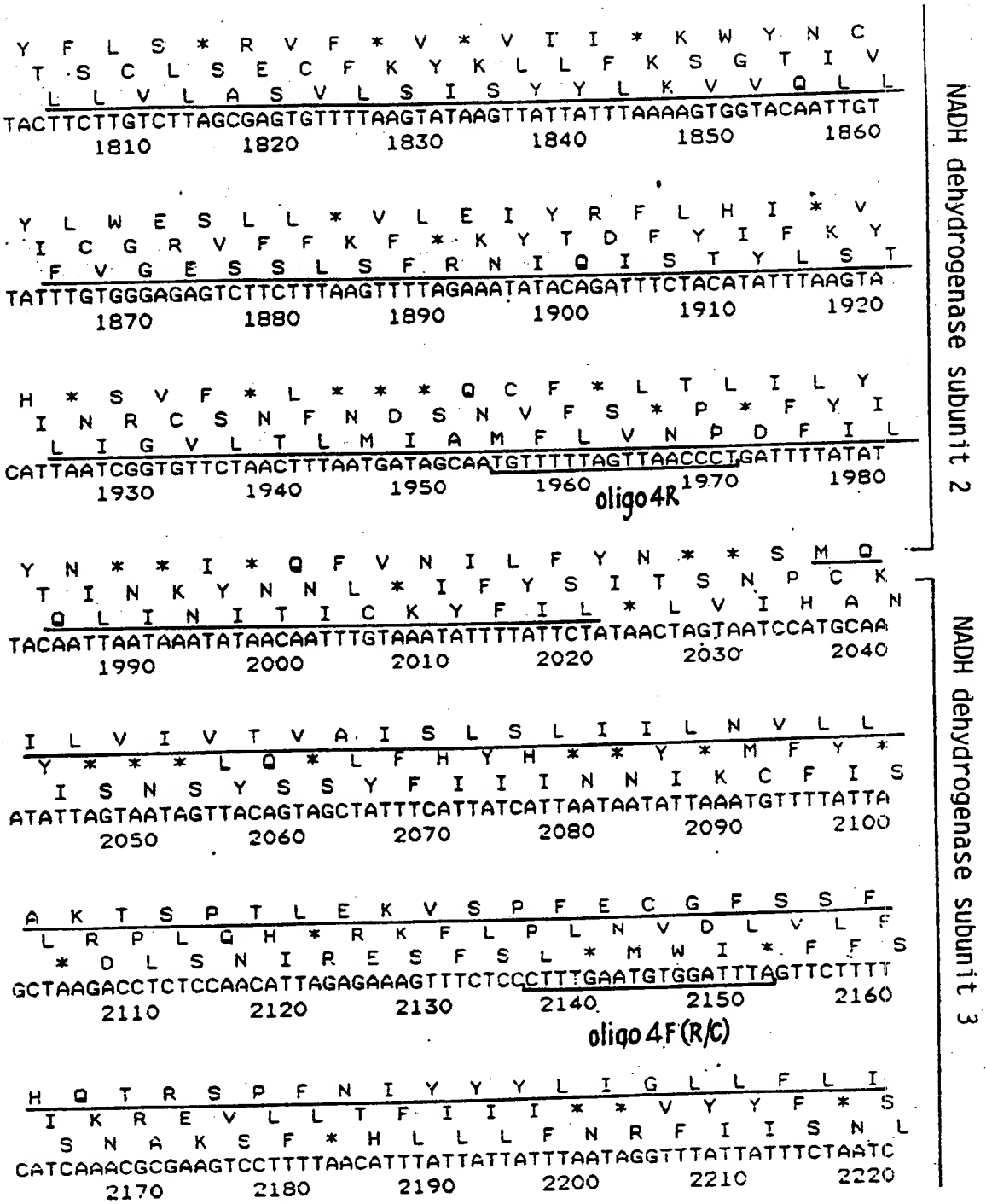
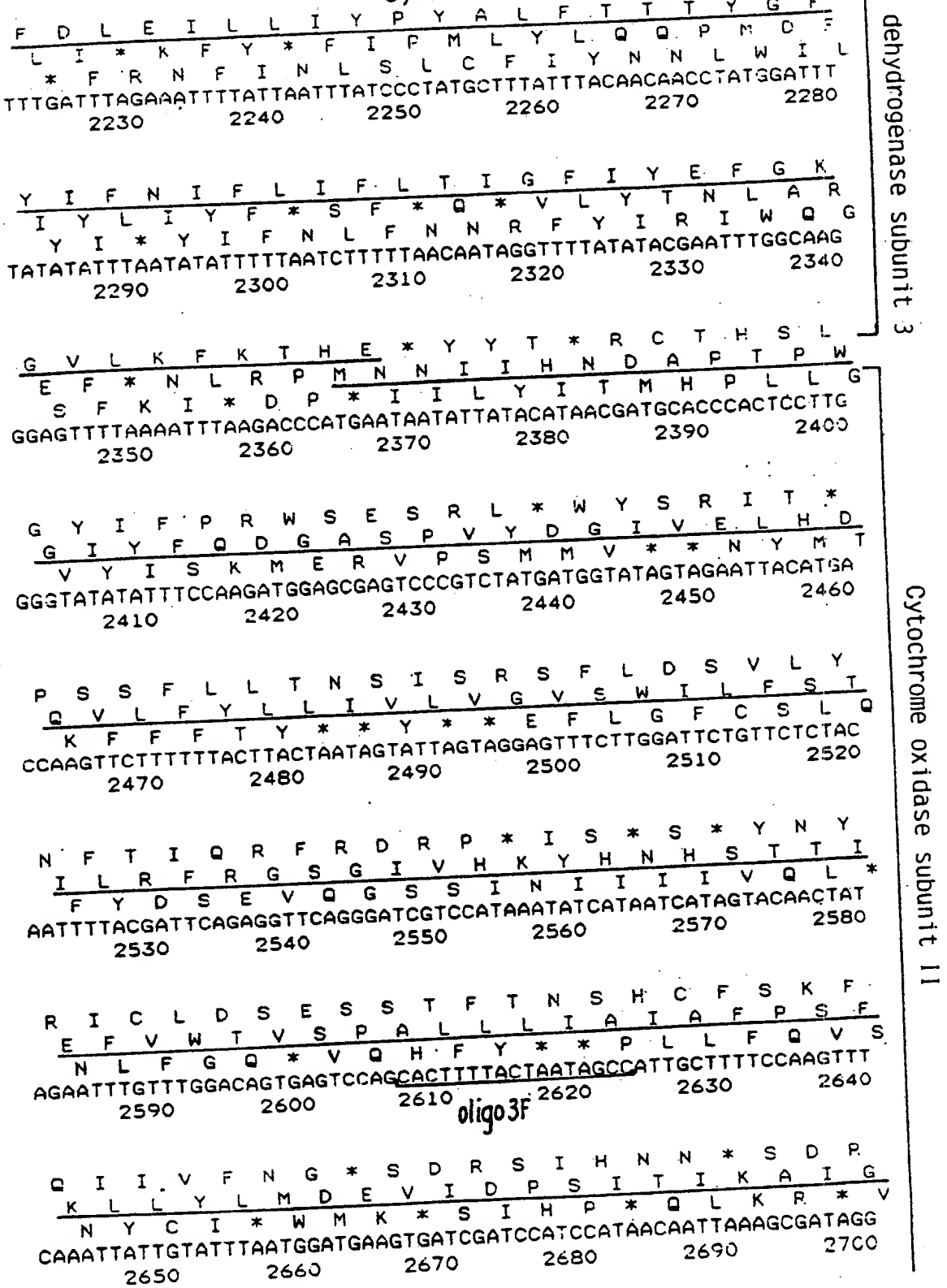
NADH dehydrogenase subunit 2

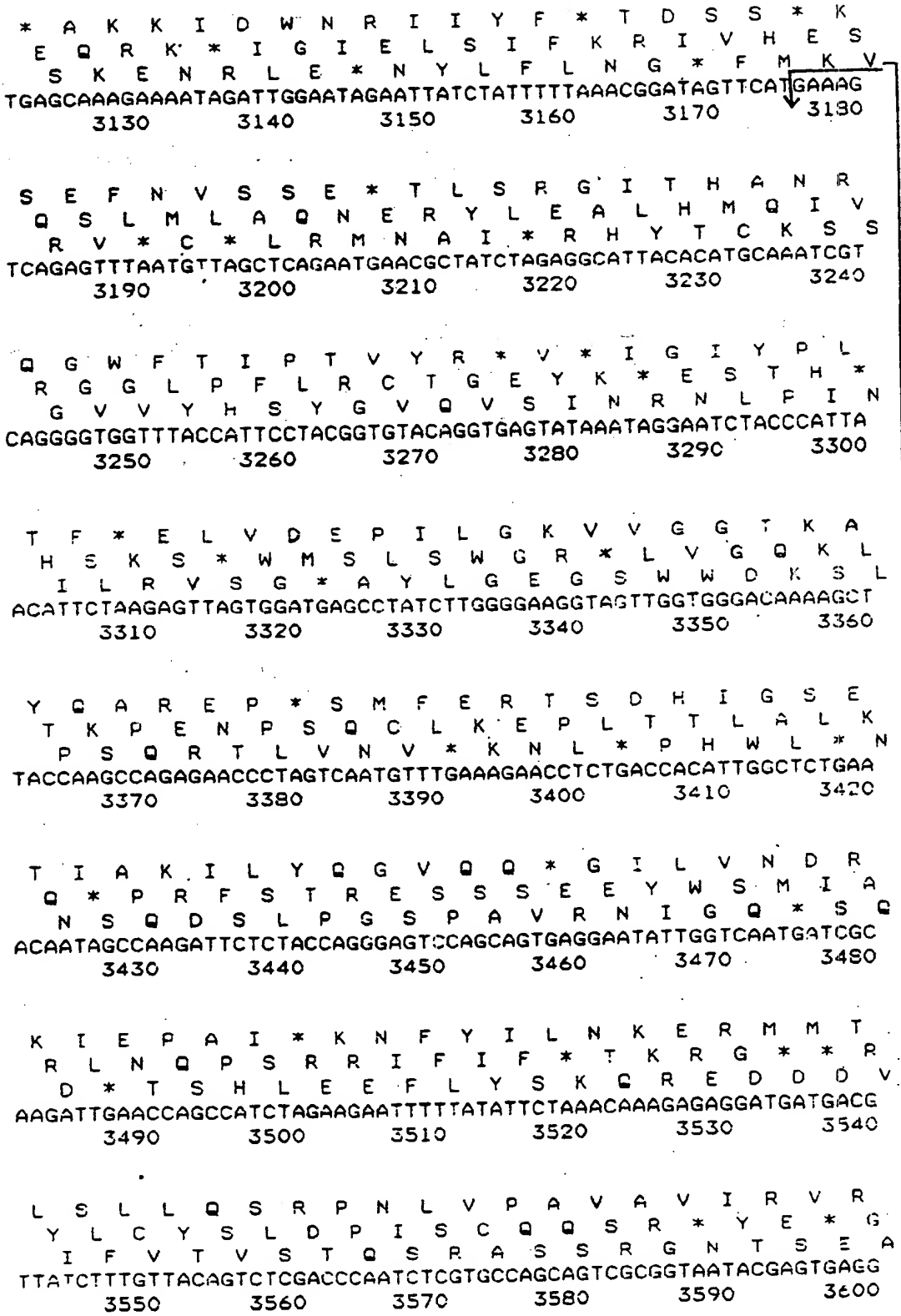

NADH dehydrogenase subunit 2
NADH dehydrogenase subunit 3
oligo 4R
oligo 4F (R/C)


NADH dehydrogenase subunit 3
Cytochrome oxidase subunit II
oligo3F

```
S  S  M  V  L  V  L  *  I  L  R  L  Y  R  Q  R  G  S  I  Y
H  Q  W  Y  W  S  Y  E  Y  S  D  Y  T  D  K  E  G  Q  S  I
 I  N  G  I  G  P  M  N  T  P  I  I  Q  T  K  R  V  N  L  *
TCATCAATGGTATTGGTCCTATGAATACTCCGATTATACAGACAAAGAGGGTCAATCTAT
      2710      2720      2730      2740      2750      2760

R  I  *  F  L  Y  V  T  H  R  P  S  *  G  G  S  I  K  T  I
E  F  D  S  Y  M  L  P  T  E  D  L  E  E  G  Q  L  R  Q  L
 N  L  I  L  I  C  Y  P  Q  K  I  L  R  R  V  N  *  D  N  *
AGAATTTGATTCTTATATGTTACCCACAGAAGATCTTGAGGAGGGTCAATTAAGACAATT
      2770      2780      2790      2800      2810      2820

R  G  *  *  P  S  L  S  S  S  E  Y  S  S  S  I  Y  Y  Y  C
E  V  D  N  R  V  L  V  P  V  N  T  P  L  R  F  I  I  T  A
 R  L  I  T  E  S  *  F  Q  *  I  L  L  F  D  L  L  L  L  L
AGAGGTTGATAACCGAGTCTTAGTTCCAGTGAATACTCCTCTTCGATTTATTATTACTGC
      2830      2840      2850      2860      2870      2880

Y  R  C  F  T  *  F  C  G  S  F  F  R  N  Q  S  G  C  E  S
T  D  V  L  H  D  F  A  V  P  S  L  G  I  K  V  D  A  S  P
 Q  M  F  Y  M  I  L  F  F  L  L  *  E  S  K  W  M  R  V  Q
TACAGATGTTTTACATGATTTTGCGGTTCCTTCTTTAGGAATCAAAGTGGATGCGAGTCC
      2890      2900      2910      2920      2930      2940

R  S  I  K  S  S  I  N  I  C  T  T  *  R  S  V  L  W  S  M
G  R  L  N  Q  V  S  T  Y  V  Q  R  E  G  V  Y  Y  G  Q  C
 V  D  *  I  K  Y  Q  H  M  Y  N  V  K  E  C  I  M  V  N  V
AGGTCGATTAAATCAAGTATCAACATATGTACAACGTGAAGGAGTGTATTATGGTCAATG
      2950      2960      2970      2980      2990      3000

*  *  T  M  W  C  I  T  *  *  Y  A  D  C  H  R  G  S  L  F
S  E  L  C  G  V  L  H  S  S  M  P  I  V  I  E  A  V  S  L
 V  N  Y  V  V  V  Y  Y  I  V  V  C  R  L  S  S  R  Q  S  L  *
TAGTGAACTATGTGGTGTATTACATAGTAGTATGCCGATTGTCATCGAGGCAGTCTCTTT
      3010      3020      3030      3040      3050      3060

R  K  I  F  I  L  V  G  *  S  I  I  H  H  S  S  I  Y  S  P
E  K  F  L  S  W  L  D  N  Q  *  S  I  I  L  P  F  T  L  R
 K  N  F  Y  L  G  W  I  I  N  N  P  S  F  F  H  L  L  S  V
AGAAAAATTTTTATCTTGGTTGGATAATCAATAATCCATCATTCTTCCATTTACTCTCCG
      3070      3080      3090      3100      3110      3120
```

oligo 3R (R/C)

Cytochrome oxidase subunit II



Small subunit rRNA

11/17

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Q A L F I I T R S K G * V G G Y E L I N
  K R Y S S L L G L K G E * V V M N L L I
    S V I H H Y * V * R V S R W L * T Y * L
CAAGCGTTATTCATCATTACTAGGTCTAAAGGGTGAGTAGGTGGTTATGAACTTATTAAT
      3610      3620      3630      3640      3650      3660

* L E S N R R I K N F G S R D E I R * Y
  N * S R I E E * R I L G V E M K S D D T
    T R V E S K N K E F W E * R * N P M I P
TAACTAGAGTCGAATCGAAGAATAAAGAATTTTGGGAGTAGAGATGAAATCCGATGATAC
      3670      3680      3690      3700      3710      3720

P K D C S W R K H Y S N Y R L T L R Y E
  Q R T A H G E S I I L I I D * H * G T K
    K G L L M A K A L F * L S T D T E V R K
CGAAAGGACTGCTCATGGCGAAAGCATTATTCTAATTATCGACTGACACTGAGGTACGAA
      3730      3740      3750      3760      3770      3780
          oligo 7R (R/C)

S I R R R K D * I P L * F M L * T M N A
  A * G G A R I R Y P C S L C C K R * M L
    H K E A Q G L D T L V V Y A V N D E C *
AGCATAAGGAGGCGCAAGGATTAGATACCCTTGTAGTTTATGCTGTAAACGATGAATGCT
      3790      3800      3810      3820      3830      3840

R N * N T L F * F L W * R L * A F H L R
  E I R I L Y F S F C G E D F K H S T * E
    K L E Y S I L V S V V K T L S I P P E K
AGAAATTAGAATACTCTATTTTAGTTTCTGTGGTGAAGACTTTAAGCATTCCACCTGAGA
      3850      3860      3870      3880      3890      3900

S T V A R L K L K T L D G H R D Q Q * S
  V L S Q G * N S K H * T V T E I S S E A
    Y C R K A E T Q N I R R S Q R S A V K H
AGTACTGTCGCAAGGCTGAAACTCAAAACATTAGACGGTCACAGAGATCAGCAGTGAAGC
      3910      3920      3930      3940      3950      3960

M L F N S I T H D K S Y H S L Y N K Y F
  C C L I R * P T T N L T T P C I I N I F
    V V * F D N P R Q I L P L L V * * I F S
ATGTTGTTTAATTCGATAACCCACGACAAATCTTACCACTCCTTGTATAATAAATATTTT
      3970      3980      3990      4000      4010      4020

P * G I V L T L R N F N * I I T Y I L M
  L K G L F * L * G T L I K * * H I Y L *
    L R D C F D F E E L * L N N N I Y T Y D
CCTTAAGGGATTGTTTTGACTTTGAGGAACTTTAATTAAATAATAACATATATACTTATG
      4030      4040      4050      4060      4070      4080
```

Small subunit rRNA

```
I S L * T Y L Y I F V I R H L N L Y * *
 * V F K L T Y I Y L * Y V I * T Y I N N
  K S L N L L I Y I C N T S S K L I L I I
ATAAGTCTTTAAACTTACTTATATATATTTGTAATACGTCATCTAAACTTATATTAATAA
      4090      4100      4110      4120      4130      4140

 * F N S Y Y * I D D I I V V N V M D S N
  N S I V I I E S M T * L L L M * W I V I
   I Q * L L L N R * H N C C * C D G * * *
TAATTCAATAGTTATTATTGAATCGATGACATAATTGTTGTTAATGTGATGGATAGTAAT
      4150      4160      4170      4180      4190      4200

 N N * I L L W V * V T L V I I T N Y Y H
  I I K Y C Y G C E * H * * * * L I I T I
   * L N I V M G V S N I S N N N * L L P L
AATAATTAAATATTGTTATGGGTGTGAGTAACATTAGTAATAATAACTAATTATTACCAT
      4210      4220      4230      4240      4250      4260

 Y K P Q * * * L I K * L N G M N D * A I
  I N H S N N N * L N N * M E * M I K Q L
   * T T V I I I D * I I K W N E * L S N *
TATAAACCACAGTAATAATAATTGATTAAATAATTAAATGGAATGAATGATTAAGCAATT
      4270      4280      4290      4300      4310      4320

 S N S L I F I T G V A W L S L V R V V K
  V I H * Y L L Q V L H G C L * F V L * N
   * F I N I Y Y R C C M A V F S S C C E M
AGTAATTCATTAATATTTATTACAGGTGTTGCATGGCTGTCTTTAGTTCGTGTTGTGAAA
      4330      4340      4350      4360      4370      4380
                oligo 2R

 C * V N S E N E R N P Y L Y L K L Y K E
  V R L I P K T N A I L I F I * N Y I K R
   L G * F R K R T Q S L S L F K T I * R G
TGTTAGGTTAATTCCGAAAACGAACGCAATCCTTATCTTTATTTAAAACTATATAAAGAG
      4390      4400      4410      4420      4430      4440

 V S F H K K E * L G * R Q V L M T L M E
  Y L S I R R N N * G E D K S S * P L W S
   I F P * E G I I R V K T S P H D P Y G V
GTATCTTTCCATAAGAAGGAATAATTAGGGTGAAGACAAGTCCTCATGACCCTTATGGAG
      4450      4460      4470      4480      4490      4500

 W A T D V P Q I F L Q R E A K M K V * A
  G L Q T C H K Y F Y K G K Q R * K S E L
   G Y R R A T N I S T K G S K D E S L S *
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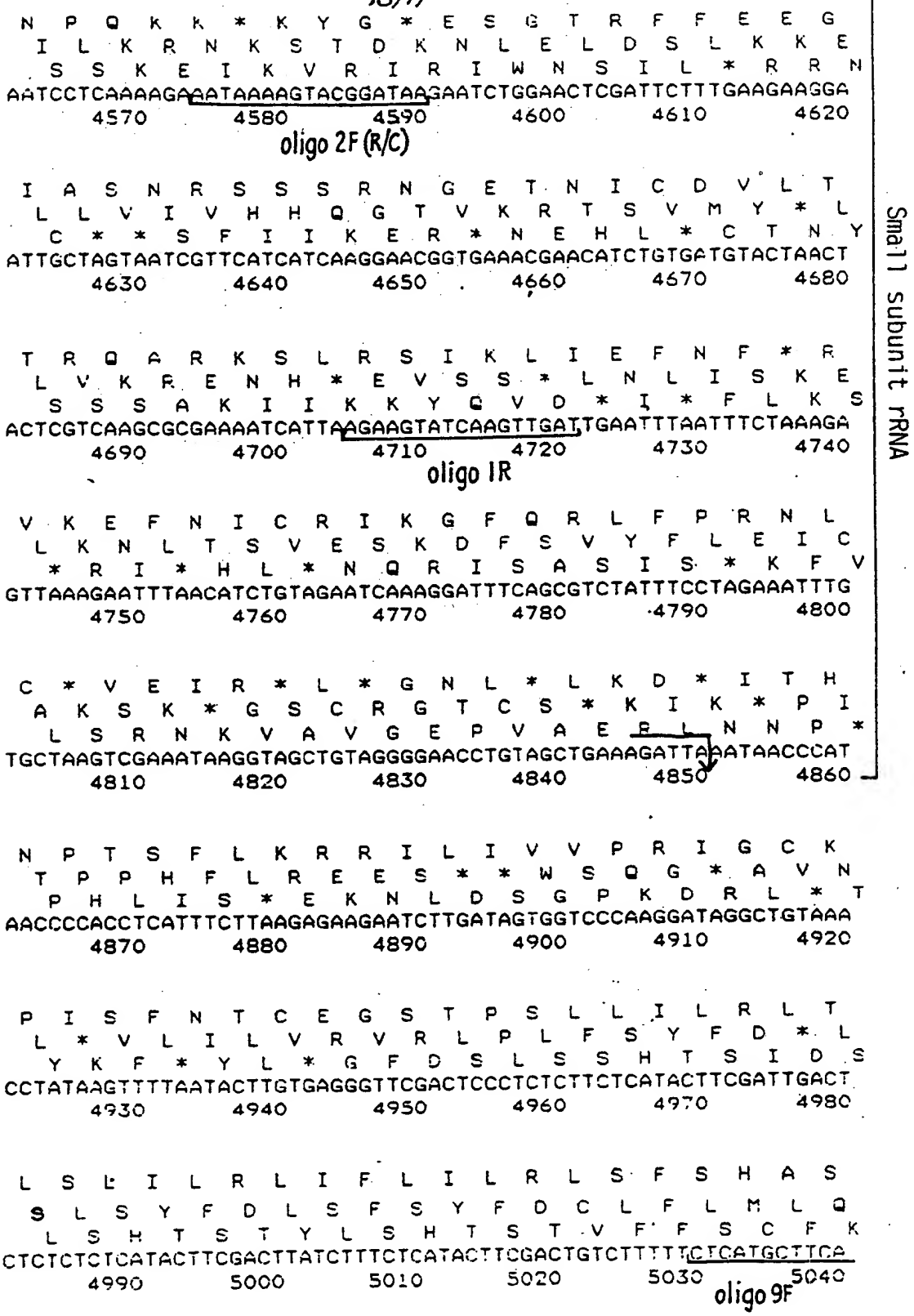
Small subunit rRNA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S S K E I K V R I R I W N S I L * R R N
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4570 4580 4590 4600 4610 4620
oligo 2F (R/C)
I A S N R S S S R N G E T N I C D V L T
L L V I V H H Q G T V K R T S V M Y * L
C * * S F I I K E R * N E H L * C T N Y
ATTGCTAGTAATCGTTCATCATCAAGGAACGGTGAAACGAACATCTGTGATGTACTAACT
4630 4640 4650 4660 4670 4680
T R Q A R K S L R S I K L I E F N F * R
L V K R E N H * E V S S * L N L I S K E
S S S A K I I K K Y Q V D * I * F L K S
ACTCGTCAAGCGCGAAAATCATTAAGAAGTATCAAGTTGATTGAATTTAATTTCTAAAGA
4690 4700 4710 4720 4730 4740
oligo 1R
V K E F N I C R I K G F Q R L F P R N L
L K N L T S V E S K D F S V Y F L E I C
* R I * H L * N Q R I S A S I S * K F V
GTTAAAGAATTTAACATCTGTAGAATCAAAGGATTTCAGCGTCTATTTCCTAGAAATTTG
4750 4760 4770 4780 4790 4800
C * V E I R * L * G N L * L K D * I T H
A K S K * G S C R G T C S * K I K * P I
L S R N K V A V G E P V A E R L N N P *
TGCTAAGTCGAAATAAGGTAGCTGTAGGGGAACCTGTAGCTGAAAGATTAAATAACCCAT
4810 4820 4830 4840 4850 4860
N P T S F L K R R I L I V V P R I G C K
T P P H F L R E E S * * W S Q G * A V N
P H L I S * E K N L D S G P K D R L * T
AACCCCACCTCATTTCTTAAGAGAAGAATCTTGATAGTGGTCCCAAGGATAGGCTGTAAA
4870 4880 4890 4900 4910 4920
P I S F N T C E G S T P S L L I L R L T
L * V L I L V R V R L P L F S Y F D * L
Y K F * Y L * G F D S L S S H T S I D S
CCTATAAGTTTTAATACTTGTGAGGGTTCGACTCCCTCTCTTCTCATACTTCGATTGACT
4930 4940 4950 4960 4970 4980
L S L I L R L I F L I L R L S F S H A S
S L S Y F D L S F S Y F D C L F L M L Q
L S H T S T Y L S H T S T V F F S C F K
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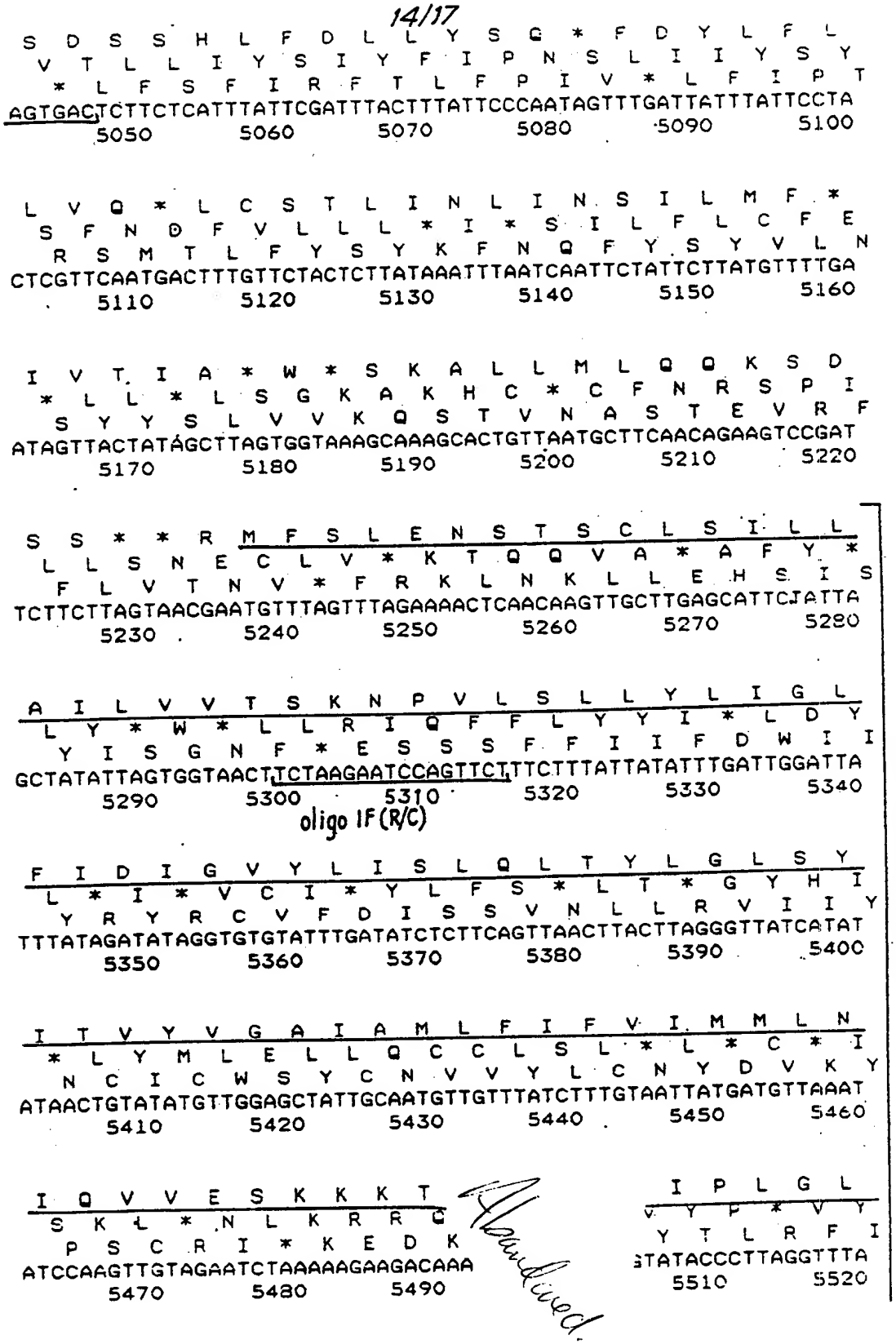
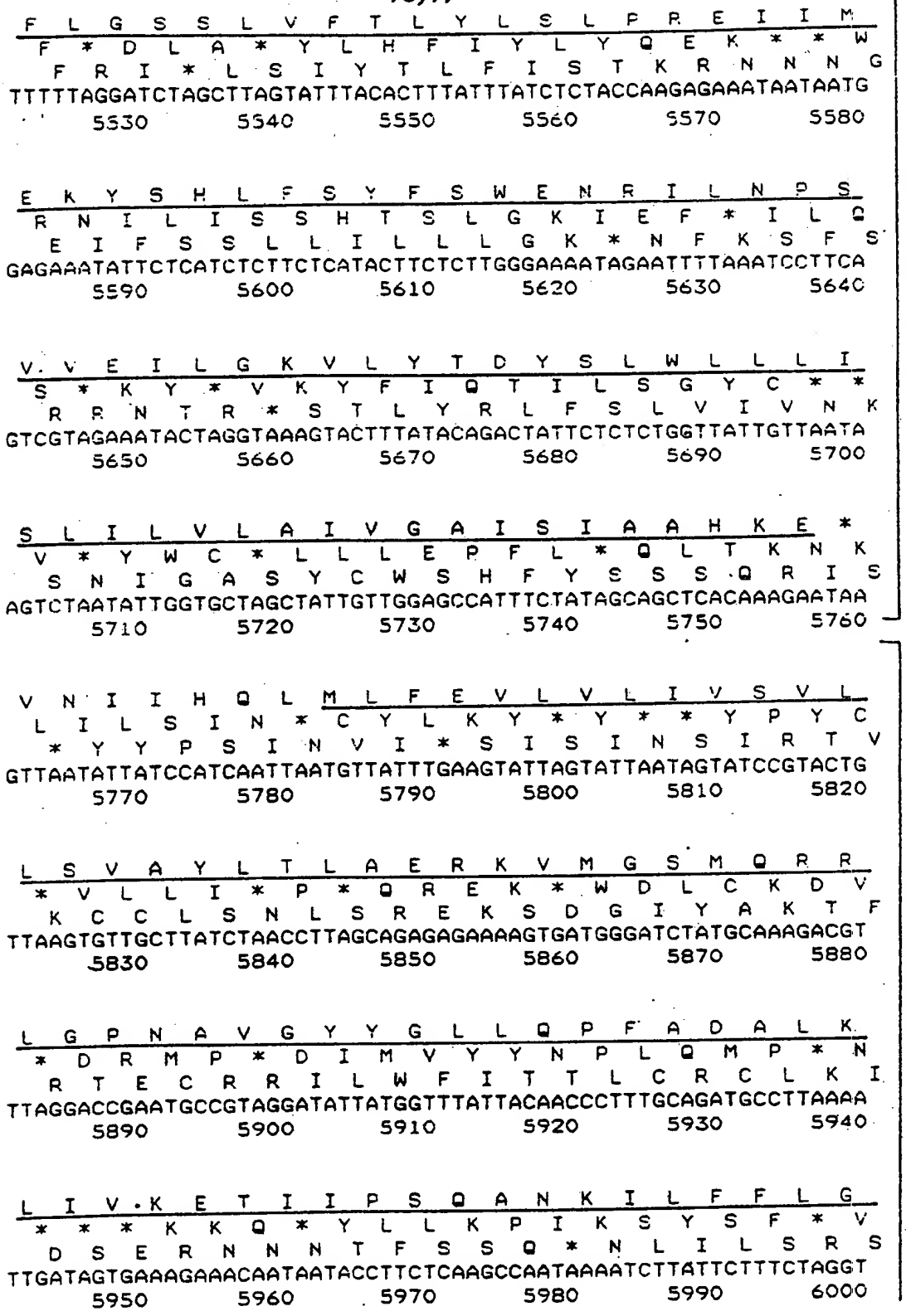
oligo 9F
Small subunit rRNA



NADH dehydrogenase 6



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F * D L A * Y L H F I Y L Y Q E K * * W
F R I * L S I Y T L F I S T K R N N N G
TTTTTAGGATCTAGCTTAGTATTTACACTTTATTTATCTCTACCAAGAGAAATAATAATG
5530 5540 5550 5560 5570 5580

E K Y S H L F S Y F S W E N R I L N P S
R N I L I S S H T S L G K I E F * I L Q
E I F S S L L I L L L G K * N F K S F S
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5590 5600 5610 5620 5630 5640

V V E I L G K V L Y T D Y S L W L L L I
S * K Y * V K Y F I Q T I L S G Y C * *
R R N T R * S T L Y R L F S L V I V N K
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5650 5660 5670 5680 5690 5700

S L I L V L A I V G A I S I A A H K E *
V * Y W C * L L L E P F L * Q L T K N K
S N I G A S Y C W S H F Y S S S Q R I S
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V N I I H Q L M L F E V L V L I V S V L
L I L S I N * C Y L K Y * Y * * Y P Y C
* Y Y P S I N V I * S I S I N S I R T V
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5770 5780 5790 5800 5810 5820

L S V A Y L T L A E R K V M G S M Q R R
* V L L I * P * Q R E K * W D L C K D V
K C C L S N L S R E K S D G I Y A K T F
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5830 5840 5850 5860 5870 5880

L G P N A V G Y Y G L L Q P F A D A L K
* D R M P * D I M V Y Y N P L Q M P * N
R T E C R R I L W F I T T L C R C L K I
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5890 5900 5910 5920 5930 5940

L I V K E T I I P S Q A N K I L F F L G
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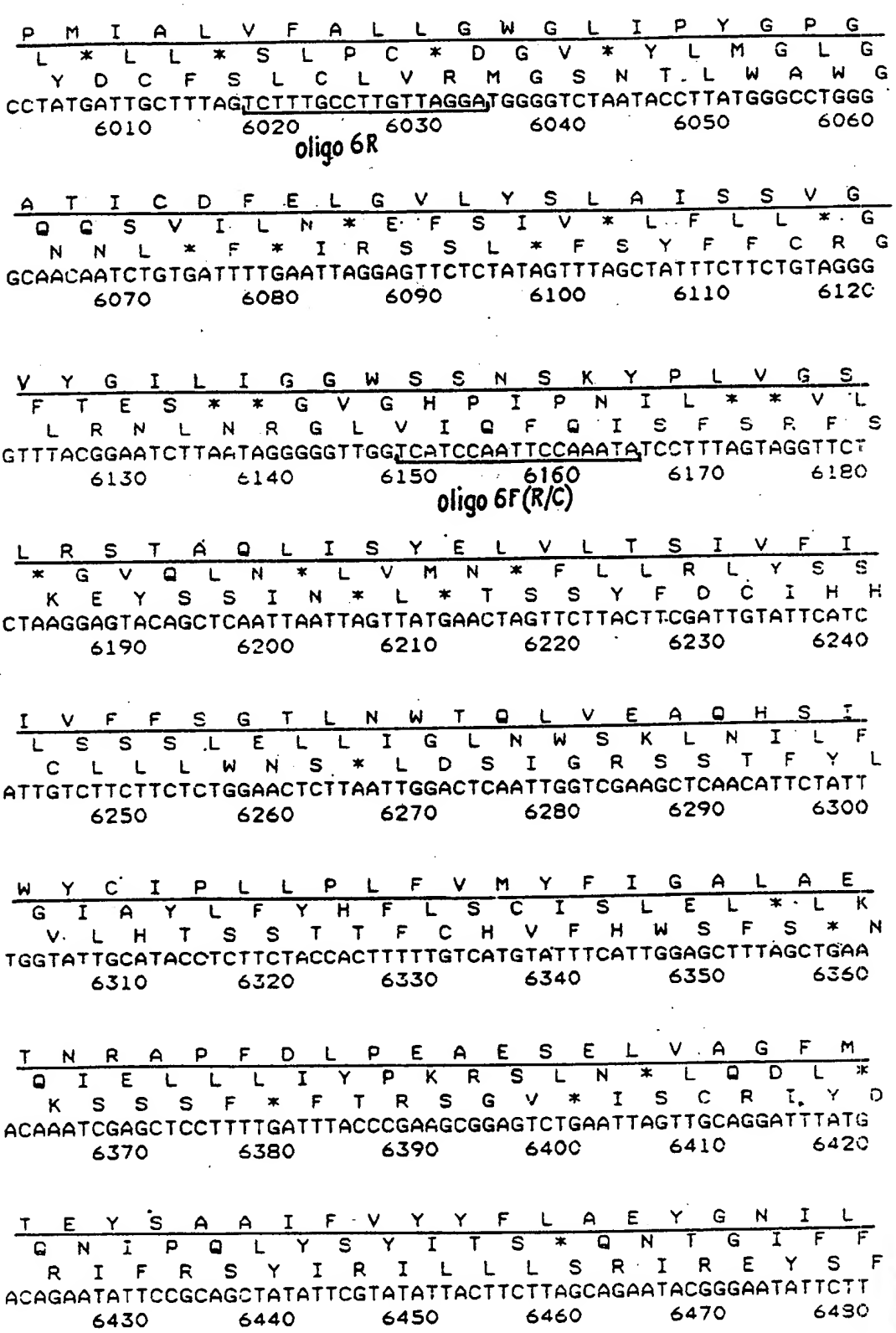
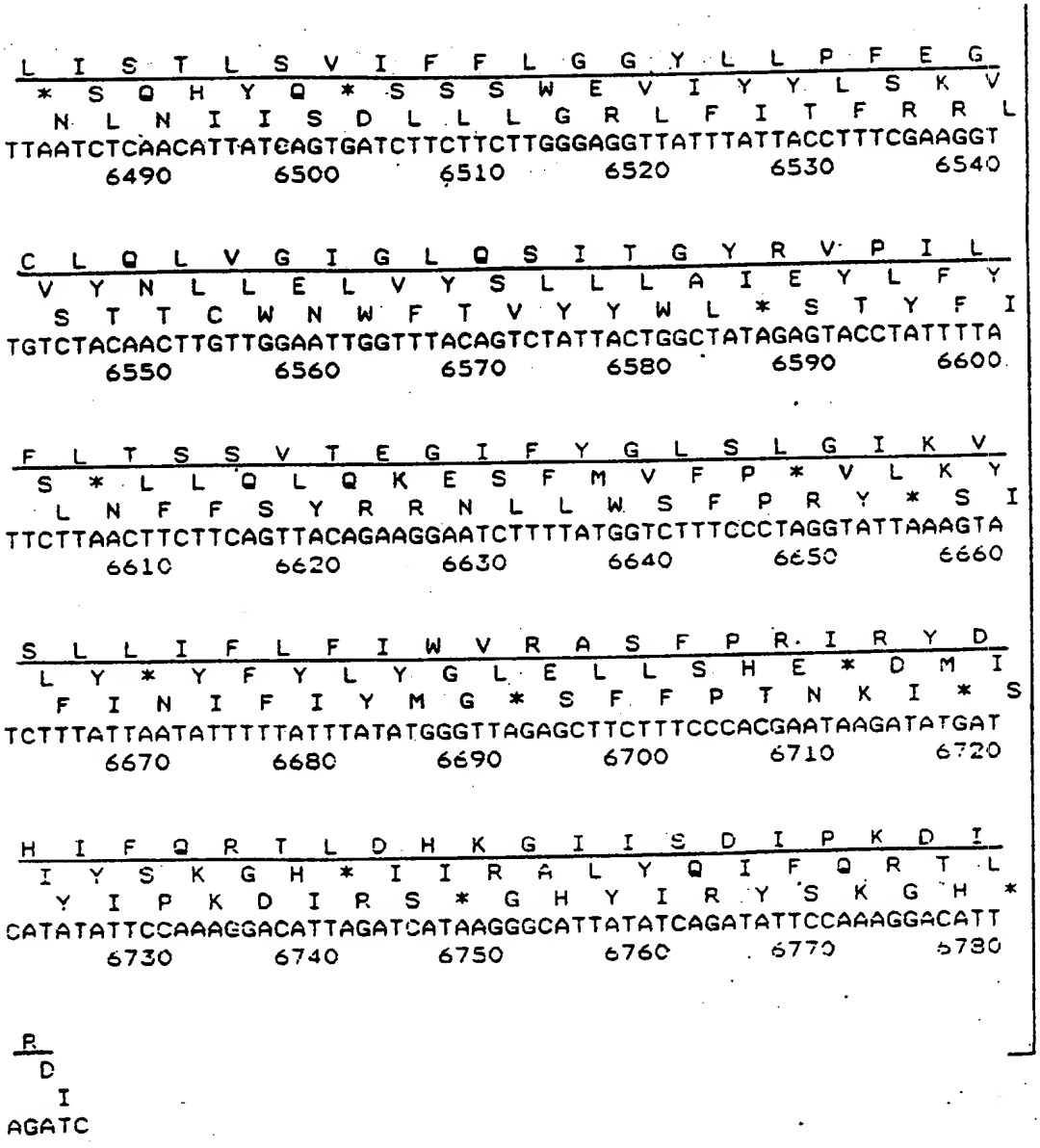


NADH dehydrogenase 1



NADH dehydrogenase 1

INTERNATIONAL SEARCH REPORT

International Applicat. No PCT/GB 91/00869

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl.5 C 12 Q 1/68 C 12 P 19/34 C 07 H 21/04
C 07 K 15/02 // C 12 N 15/11

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols		
Int.Cl.5	C 12 Q C 07 K	C 12 P A 61 K	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
X	EP,A,0327390 (ISIS INNOVATION LTD) 9 August 1989, see figures 2,3, which correspond to figure 3 of application, nucleotides 1-89,90-539 respectively (cited in the application)	2
Y	---	4,5,6,9
P,X	THE LANCET, vol. 336, no. 8713, August 1990, A.E. WAKEFIELD et al.: "Detection of Pneumocystis carinii with DNA amplification", pages 451-453, see the whole article	2,4-8
P,Y	--- WO,A,9013669 (XYTRONYX INC.) 15 November 1990, see the whole document	4,5,6,9
	--- -/-	

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
09-09-1991	28. 10. 91
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Mme. M. van der Drift

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
A	CHEMICAL ABSTRACTS, vol. 111, no. 3, 3 July 1989, page 205, abstract no. 2066k, (Columbus, Ohio, US), A.E. WAKEFIELD et al.: "Cloning of DNA from Pneumocystis carinii", & J. PROTOZOOL. 1989, 36(1), 55-7S	
P,X	MOLECULAR AND BIOCHEMICAL PARASITOLOGY, vol. 45, no. 1, January 1991, Elsevier Science Publishers B.V. (Biomedical Division), K. SINCLAIR et al.: "Pneumocystis carinii organisms derived from rat and human hosts are genetically distinct", pages 183-184, see the whole article	1-8
P,X	MOLECULAR AND BIOCHEMICAL PARASITOLOGY, vol. 43, no. 1, November 1990, Elsevier Science Publishers B.V. (Biomedical Division), A.E. WAKEFIELD et al.: "Amplification of mitochondrial ribosomal RNA sequences from Pneumocystis carinii DNA rat and human origin", pages 69-76, see the whole article	1-8

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO. GB 9100869 SA 48160

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 17/10/91
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP-A- 0327390	09-08-89	JP-A- 2005899	10-01-90
WO-A- 9013669	15-11-90	AU-A- 5671190	29-11-90